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'Giardia muris': Ultrastructural Analysis of 'In vitro' Excystation

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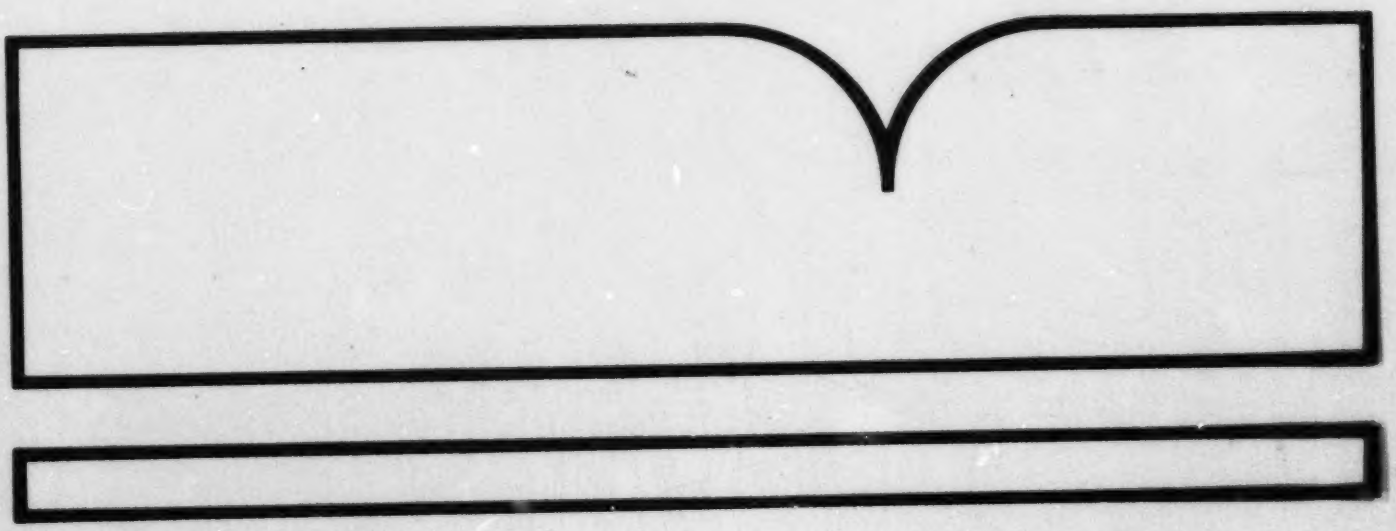

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16. ABSTRACT

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Giardia muris: Ultrastructural Analysis of *in Vitro* Excystation

JAMES R. COGGINS AND FRANK W. SCHAEFER III

Department of Biological Sciences, University of Wisconsin–Milwaukee, Wisconsin 53201, U.S.A., and Health Effects Research Laboratory, United States Environmental Protection Agency, 26 West St. Clair Street, Cincinnati, Ohio 45268, U.S.A.

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COGGINS, J. R., AND SCHAEFER, F. W., III. 1986. *Giardia muris:* Ultrastructural analysis of *in vitro* excystation. *Experimental Parasitology* 61, 219–228. *Giardia muris* cysts were examined by transmission electron microscopy before treatment, after induction, and at timed intervals during the incubation phase of *in vitro* excystation. Untreated *G. muris* cysts had a thick cyst wall composed of a fibrous outer wall and a thin, electron-dense inner membrane which extended from the trophozoite plasma membrane. The cytoplasm was devoid of endoplasmic reticulum, Golgi bodies, and mitochondria. Numerous large vacuoles were present within the ectoplasm just beneath the plasma membrane in untreated cysts. Following induction these cysts lacked ectoplasmic vacuoles. Concurrently, numerous membrane bound vesicles were seen in the peritrophic space closely adhering to the surface of the trophozoite. These vesicles appear to be of cytoplasmic origin. The cytoplasm of fully excysted trophozoites lacked ectoplasmic vacuoles but displayed well-developed ribbons of microtubular bodies, probably precursors of ventral disk, lateral flange, and median bodies and also contained extensive granular endoplasmic reticulum. No more than two nuclei were observed within each organism. The earliest excysted organisms were observed 0–5 min after incubation had begun and most organisms had excysted within 10 min. Cytokinesis occurred only after excystation was complete. © 1986 Academic Press, Inc.

INDEX DESCRIPTORS: *Giardia muris;* Protozoa, parasitic; Cyst; Excystation; Transmission electron microscopy; Cultivation, *in vitro;* Mouse, CF-1.

INTRODUCTION

Giardia sp. is a major cause of enteric disease worldwide. Numerous outbreaks of waterborne giardiasis have been reported in the United States in the past few years (Craun *et al.* 1984; Lippy 1981) and giardiasis is of increasing public health concern (Erlandsen and Meyer 1984).

Although several species within the genus are recognized (Filice 1952; Grant and Woo 1978), few distinct morphological differences exist (Meyer and Jarroll 1980). Host specificity is not absolute (Meyer and Radulescu 1979) and cross-infectivity between humans and a variety of other animals is known to occur (Woo 1984; Davies and Hibler 1979). *Giardia muris,* an intestinal protozoan parasite of rodents, has been utilized in a number of studies as a model of human giardiasis (Robert-Thomson *et al.* 1976; Owen 1980). The validity of the murine model as a basis for human giardiasis has been questioned recently (Meyer and Schaefer 1984).

Even though *Giardia* sp. was first described over 300 years ago, comparatively little is known of its physiology and biochemistry (Visvesvara 1982). Methods for *in vitro* cultivation and *in vitro* excystation are important in order to produce reliable and large quantities of the parasites. Unfortunately, techniques successful for the excystation of *G. lamblia* have not proven reliable for *G. muris* (Meyer and Schaefer 1984; Schaefer *et al.* 1984). Excystation of a *Giardia* sp. was first described by Hegner (1925, 1927a, b). Methods for *in vitro* excystation of *G. lamblia* have been reported

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recently by Bingham and Meyer (1979) and of *G. muris* by Schaefer *et al.* (1984).

The ultrastructure of *Giardia* sp. trophozoites has been described by several workers using transmission (Friend 1966; Nemanic *et al.* 1979; Owen 1980) and scanning electron microscopy (Mueller *et al.* 1973; Erlandsen 1974; Gordon *et al.* 1977; McKee *et al.* 1978; Owen *et al.* 1979). However, limited attention has been paid to the cyst stage of the parasite (Sheffield and Bjorvatn 1977; Sheffield 1979; Luchtel *et al.* 1980; Owen 1980; Coggins and Schaefer 1984). The structure of the trophozoite and cyst has been reviewed recently by Feely *et al.* (1984).

Light microscopy has been used to describe *in vitro* excystation of *G. lamblia* (Bingham and Meyer 1979) and *G. muris* (Meyer and Schaefer 1984). Coggins and Schaefer (1984) reported recently on the scanning electron microscopy of *G. muris* excystation *in vitro*.

Materials and Methods

Giardia muris cysts were obtained from female Swiss albino mice (CF-1) (Coggins and Schaefer 1984; Schaefer *et al.* 1984). Twenty mice were infected *per os* each week with 1000 purified *G. muris* cysts suspended in 0.2 ml distilled water. Feces were collected overnight from 60 mice between 7 and 28 days after infection. The feces were homogenized in dilute Tween 20 [0.01% (v/v) in distilled water] on a magnetic stir plate and passed successively through 10-, 20-, 40-, and 60-mesh sieves. After concentration of the sieved feces by centrifugation at 500*g* for 5 min, the pellet was resuspended in 420 ml of the dilute Tween 20 and floated two times on 1 *M* aqueous sucrose. Each float was followed by three washes in the dilute Tween 20 at 500*g* for 5 min.

The *G. muris* cyst preparation was further purified by sedimentation at unit gravity on a continuous Percoll gradient which ranged in density from 1.01 to 1.03 g/ml (Sauch 1983). The gradient was contained in a cylinder 7 cm high by 11 cm diameter and was roughly 6 cm high in the cylinder. Between 2 and 3 $\times$ 10^8 *G. muris* cysts in 20 ml dilute Tween 20 were layered on top of the Percoll gradient. After a sedimentation time of 2.5 hr, the *G. muris* cyst band was removed from the Percoll by washing two times through the dilute Tween 20 (300*g*, 2 min). The *G. muris* cyst preparation appeared free of contamination when examined by phase microscopy. Cysts were stored in distilled water at 4 C and used within 5 days of isolation.

Excystation of the *G. muris* cysts was accomplished using the procedure of Schaefer *et al.* (1984). This procedure is divided into three steps, including (1) induction, (2) wash, and (3) incubation. Approximately 13 $\times$ 10^6 *G. muris* cysts contained in 0.5 ml distilled water were placed in a 15-ml screwcap tube. Reducing solution (Meyer 1976) and 0.1 *M* sodium bicarbonate (5 ml each) were added in that order. The cap to the tube was screwed on quickly to prevent loss of the CO_2 generated; the tube was vortexed and incubated 30 min in a 35 C water bath. The *G. muris* cysts were then centrifuged in a swinging bucket rotor for 2 min at 300*g*. After aspiration of the supernatant, the *G. muris* cysts were resuspended in a wash solution of 10 ml trypsin/Tyrode's solution [0.5% (w/v) trypsin dissolved in 1X Tyrode's solution, pH 8] by vortexing. The *G. muris* cysts were removed from the trypsin/Tyrode's wash by centrifugation at 300*g* for 2 min and were resuspended in 10 ml trypsin/Tyrode's solution. The tube was then returned to the 35 C water bath and incubated for varying lengths of time as indicated below.

For transmission electron microscopy, 0.1 ml aliquots of cyst suspension were sampled 0, 5, 10, 15, and 30 min after incubation of the cysts had begun. Each aliquot was fixed in an excess of phosphate-buffered 4% glutaraldehyde and collected onto 47-mm, 3.4-μm pore-size Nucleopore membranes which had been pretreated in 0.01% (w/v) polylysine (Sigma, St. Louis, MO, USA). The membranes were held in a Millipore filter holder using minimal vacuum. Additional glutaraldehyde was added, the holder was capped, and the suspension was fixed for a total of 2 hr at room temperature. After washing, the suspension was postfixed in phosphate-buffered 1% osmium tetroxide for 1 hr. Subsequent dehydration was through an ascending ethanol series. The parasite laden membrane was quickly removed from the holder and was either cut into small pieces and embedded or, if possible, the parasite layer was removed from the membrane and embedded in a low viscosity embedding medium (Spurr 1969). At no time were the membranes or pellets allowed to dry.

Silver to gold sections were cut and stained with uranyl acetate and lead citrate. Sections were viewed using a Hitachi 11B transmission electron microscope operated at 75 kV. In a subsequent experiment, cysts were processed as above but stained during dehydration using uranyl acetate. This additional step appeared to enhance visualization of the outer cyst wall.

Results

Ultrastructure of untreated *Giardia muris* cysts observed in the present experi-

ments was similar to results of previous reports (see Feely *et al.* 1984). The outer cyst wall appeared to be of uniform thickness (approximately 0.3–0.4 μm) and composition with no obvious areas of weakness or distinctive morphology, indicative of an area where the trophozoite would exit, in either transverse (Fig. 1) or longitudinal (Fig. 2) section. The cyst wall was composed of two distinct layers. The outer cyst wall consisted of fibrillar elements interspersed with small dense granules. The outer surface appeared smooth with bacteria occasionally seen adjacent or attached to the cyst surface (Fig. 1). The inner dense membranous portion of the cyst wall was always observed directly adjacent to the fibrillar region of the wall (Fig. 3). The inner cyst membrane was observed to be continuous with the plasma membrane of the trophozoite (Fig. 4). In a few of the sections observed the trophozoite cytoplasm appeared to abut the outer fibrillar cyst wall directly (Fig. 4).

The encysted parasite either adhered closely to the wall or was separated by open areas that will here be termed the peritrophic space (Fig. 3). Much more open area was seen at the posterior end of the parasite than at the anterior (Fig. 2). In the present experiment, no Golgi bodies, mitochondria, or endoplasmic reticulum were observed within the untreated encysted trophozoite. Untreated cysts did contain large numbers of membrane bound vacuoles located within the ectoplasm; no connection between vacuoles was observed. While some vacuoles contained a dense, cytoplasmal inclusion, most appeared devoid of contents (Fig. 3). Little glycogen and no other recognizable endocellular constituents occurred in the ectoplasm. In contrast, abundant glycogen, microtubular like material presumed to be median bodies, flagellar axonemes, and microtubular ribbons in various states of assembly were common in the endoplasmic region of encysted parasites.

Cytoplasmic changes in cysts began to appear as soon as the cyst su
placed in the trypsin/Tyrode
solution. Although a few org
excystment immediately after
in this medium, probably du
wash, most trophozoites were
until 5–15 min after incubatio
Almost all organisms had ex
min after incubation. Those u
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tion appeared abnormal.

The morphology of parasit
stages of emergence contra
from that of untreated encys
Ectoplasmic vacuoles were ra
after chemical treatment had
tensive network of endoplas
was first seen in these activat
5). Emerging organisms wer
by a loose network of fibrou
that of cyst walls (Figs. 6, 7).
membrane elements were ob
this stage. While ectoplas
were rare in excysting para
number of smaller, membran
icles containing an electron
occurred on the outer surfa
membrane (Fig. 6). These v
greatly in size and appea
through exocytosis from the
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vesicles pinching off from th
(Fig. 7).

Newly excysted trophozo
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manents of cyst wall materi
cysted trophozoites displaye
oped cytoplasm with areas
ribbons, the precursor to th
and lateral flange (Figs. 8, 9)
regularly arranged microtu
with dorsally extending dens
a prominent feature of newl
muris trophozoites (Fig. 8).
toplasmic elements of exc
zoites included flagellar ax
formed nuclei, and extensiv
doplasmic reticulum (Fig. 9).

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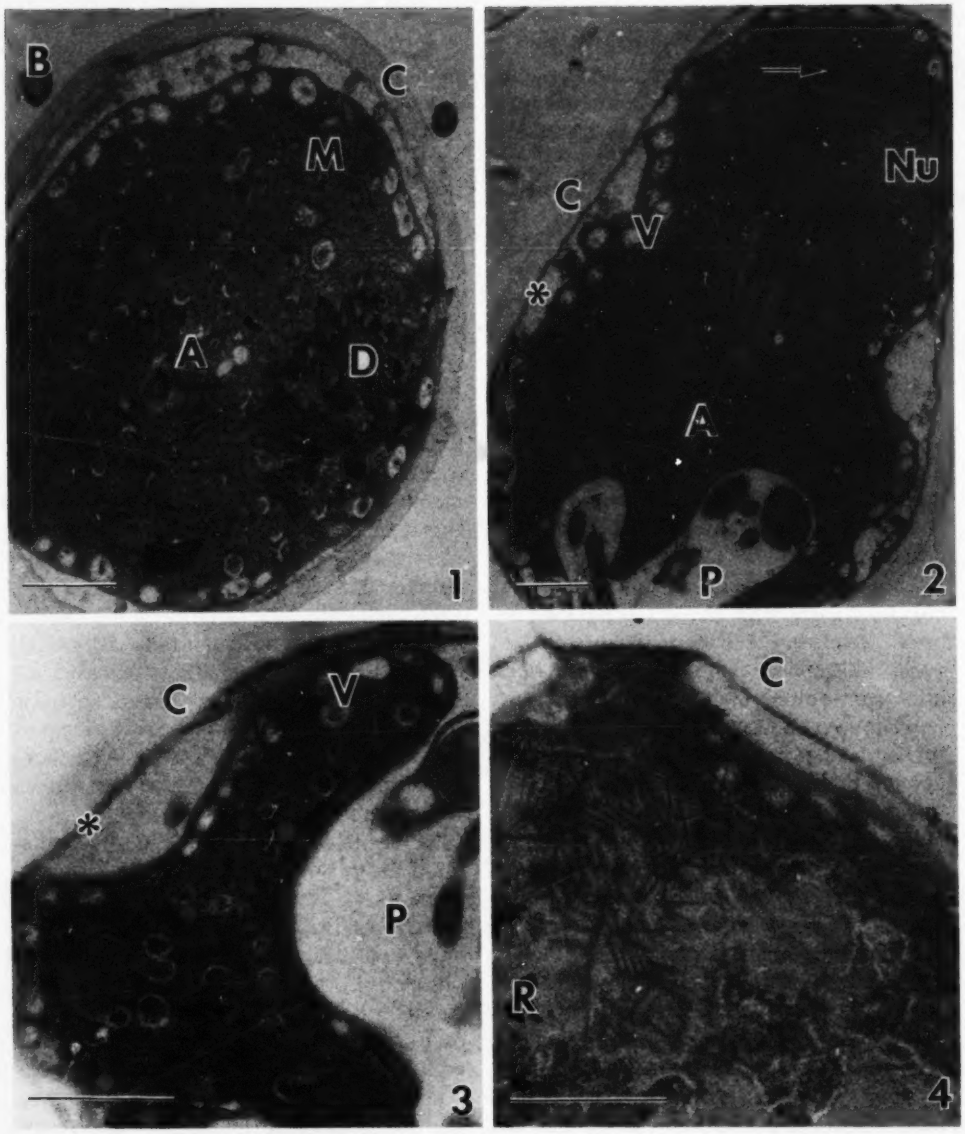
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vitro excystation until the organisms were free of all fibrous cyst wall elements (Fig. 10). Cytokinesis appeared to occur 15–30 min after incubation had begun. No external vesicles were seen around these cells. Additionally, large vacuoles were rarely seen within the cytoplasm. In marked contrast to untreated organisms, newly formed daughter trophozoites contained marked quantities of granular endoplasmic reticulum and lacked intracellular vacuoles or external vesicles (Fig. 10). The rest of the endocellular morphology in newly excysted trophozoites appeared similar to that in encysted, *in vivo* and *in vitro* cultured trophozoites.

Discussion

This report and its predecessor represent the first attempt to analyze the important process of the excystation of a *Giardia* species using scanning and transmission electron microscopy. Excystation of *Giardia* was first described by Hegner (1925, 1927a, b). The *in vitro* excystation of a *Giardia* sp. was described by Bingham and Meyer (1979) using light microscopy. Unfortunately, the excystation and culture techniques used successfully with *Giardia lamblia* do not work well for *Giardia muris*. Schaefer *et al.* (1980) developed a technique of excysting *G. muris* which is a modification of the Bingham–Meyer procedure. High rates of excystation were reported using this method. Coggins and Schaefer (1984) followed the *G. muris* excystation process using scanning electron microscopy and confirmed the high excystation rates and analyzed ultrastructural morphology of the excystation process. Unfortunately, the scanning electron microscopy results obtained by these workers gave no indication of events occurring within the cyst or actively excysting parasite. Consequently, the current report analyzed events within the excysting parasite and newly formed daughter trophozoites.

The ultrastructure of the cyst wall seen in the present study did not differ significantly from prior descriptions given by other workers (Sheffield 1979). The outer wall appeared of uniform thickness and composition with no indication of any particular area where the enclosed parasite would exit. There did not appear to be any structure similar to an operculum or any weak area of the wall. We feel that excystation must be initiated by enzymatic action from within the cyst, triggered by proper environmental conditions, and not strictly by mechanical means. Although Filice (1952) speculated that the pressure and flexing of the flagella provided the force necessary to break the cyst wall, we feel that this is unlikely. Although we previously showed apparently active flagella during excystation (Coggins and Schaefer 1984), they appeared only to enlarge the

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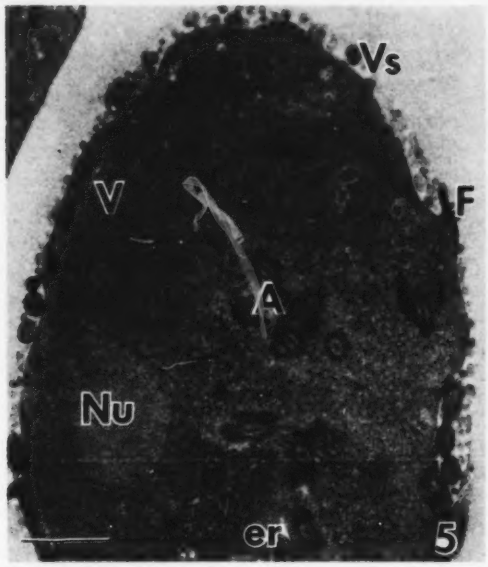
Fig. 1. Untreated cyst of *Giardia muris* showing cyst wall (C) with adherent bacteria (B). Dense ribbons represent precursors of the adhesive disk (D); the unordered series of microtubules the median body (M). Flagellar axonemes (A) occupy the medullary cell region. Scale bar indicates 1.0 μm.

Fig. 2. Longitudinal section of untreated *Giardia muris*. The outer cyst wall (C) is bounded closely on the interior by a dense inner membrane (asterisk). The peritrophic space (P) is visible at several areas around the organism but is most pronounced at the posterior region of the cyst. Note the large number of ectoplasmic vacuoles (V). Also seen are nuclei (Nu), axonemes (A), kinetosome (arrow). Scale bar indicates 1.0 μm.

Fig. 3. Untreated *Giardia muris* cyst. The dense inner membrane (asterisk) is closely applied to the outer cyst wall (C). An extensive peritrophic space (P) is present. The ectoplasmic cytoplasm contains large numbers of vacuoles (V) but is devoid of other cellular organelles. Scale bar indicates 1.0 μm.

Fig. 4. *Giardia muris* after induction. Cytoplasm of the enclosed trophozoite directly abuts the outer cyst wall (C). The continuity of the trophozoite plasma membrane with the inner membrane of the cyst wall is apparent. Note the microtubular ribbons (R) within the cytoplasm. Scale bar indicates 1.0 μm.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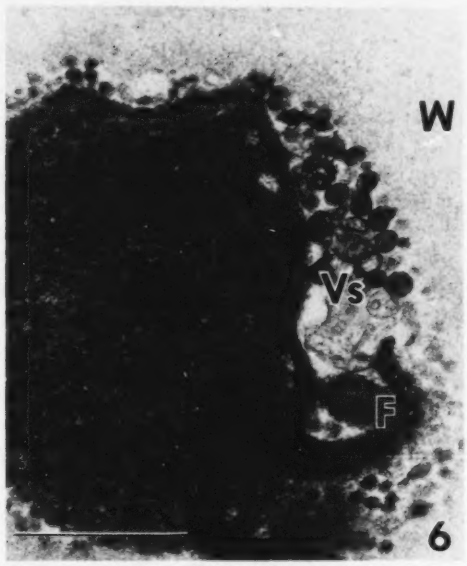
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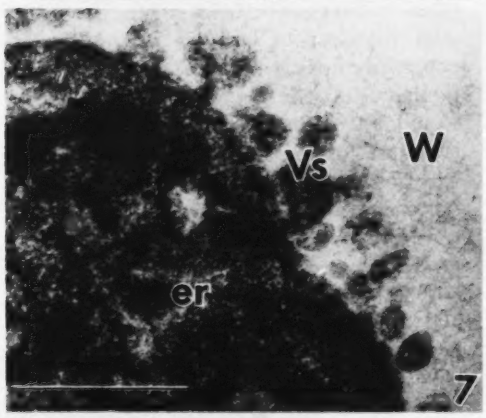
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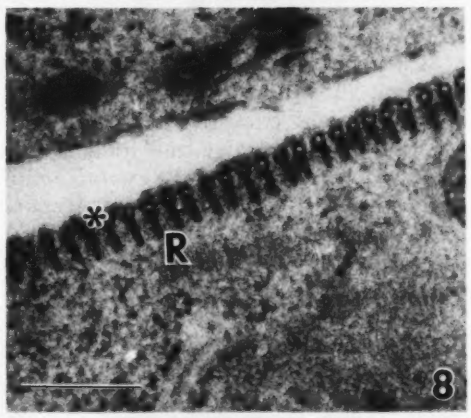
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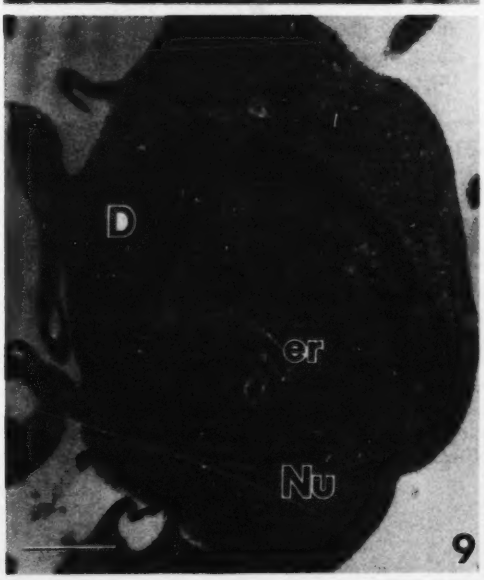
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opening after the trophozoite had un to exit. Current information concerning the presence of lysosomal enzymes dmark 1980; Feely 1982) within cysts g with evidence of mucous material secreted during excystation (Bingham and yer 1979; Coggins and Schaefer 1984) irectly supports this enzymatic hypothesis. No polarity of the cyst was observed; however, as reported earlier, the enclosed trophozoite always appeared to exit posterior end first (Coggins and aefer 1984). This finding has been reported by other workers who observed excysting *Giardia* using light microscopy (Filice 1952).

he cyst wall is obviously important as a protective structure. The outer wall was composed mainly of fibrous elements interspersed with small discrete granules. Sheffield (1979) speculated that this layer acted to impede passage of large molecules into the cyst. The inner cyst membrane was first noted by Soloviev and Shentsov (1970). To our knowledge, this inner cyst membrane has not been reported as continuous with the trophozoite plasma membrane by recent workers. We agree that this layer may control passage of small molecules through the cyst wall since it is a plasma membrane. We also observed that the inner cyst membrane appears to be an extension of the plasma membrane of the enclosed parasite. Additionally, the cytoplasm of the enclosed organism appeared to abut the cyst wall at the juncture of the cyst wall and trophozoite plasma membranes directly. These observations agree with the report of Sheffield (1979). The possible existence of this connection raises interesting questions as to the transport of elements from the excysting parasite and transfer of molecules in and out of the cyst. This apparent connection between trophozoite and the cyst wall was found in the anterior end of the trophozoite, though this could not be determined conclusively.

Although these experiments were conducted using laboriously purified cyst preparations, bacteria were occasionally observed on the surface of untreated cysts. Nevertheless, there was no evidence of endosymbiotic bacteria within the cyst as has been reported by other workers (Nemanic *et al.* 1979; Radulescu *et al.* 1982). While the significance of bacterial endosymbionts

FIG. 5. *Giardia muris* trophozoite initial incubation. Note the lack of an intact cyst wall. Few vacuoles (V) occur within the cytoplasm. A number of vesicles (Vs) surround the parasite. Cellular constituents include an extensive network of granular endoplasmic reticulum (er), nucleus (Nu), and flagellar axonemes (A). The adhesive disk and lateral flange (F) appear partially organized at this point in the excystation process. Scale bar indicates 1.0 μm.

FIG. 6. *Giardia muris* trophozoite 15 min after incubation. A network of loosely packed fibers (W) representing remnants of the cyst wall surround the organism. Note the lack of a cyst wall inner membrane and the large number of vesicles (Vs) on the cell surface. The lateral flange (F) appears well organized. Scale bar indicates 1.0 μm.

FIG. 7. *Giardia muris* trophozoite 15 min after incubation. Several vesicles (Vs) in the process of exocytosis are shown while others line the cell surface. Note the profiles of endoplasmic reticulum (er). Scale bar indicates 0.5 μm.

FIG. 8. *Giardia muris* trophozoite 15 min after incubation. An apparently well-organized adhesive disk is composed of a regular series of microtubules (asterisk) located just beneath the plasma membrane with attached, dorsally oriented dense ribbons (R). Scale bar indicates 0.25 μm.

FIG. 9. *Giardia muris* trophozoite 30 min after incubation. Few vacuoles or cellular organelles are present except for endoplasmic reticulum (er) and the nucleus (Nu). Note the absence of any surrounding fibers. Concentric ring arrangement of the adhesive disk (D) is shown. Scale bar indicates 1.0 μm.

FIG. 10. *Giardia muris* 30 min after incubation. Formation of daughter trophozoites by cytokinesis is shown. Adhesive disk (D), lateral flange (F), nuclei (Nu), and axonemes are present. Few vacuoles are noted at this stage. Scale bar indicates 1.0 μm.

remains enigmatic, they apparently occur sporadically or only in certain *Giardia* strains. Nemanic *et al.* (1979) speculated that the pathogenecity of the trophozoite may be altered by the presence of endosymbionts. Furthermore, Feely *et al.* (1984) have reported virus-like particles within *Giardia muris* trophozoites. Although they were not observed in the present study, endosymbionts may be significant in those strains of *Giardia* sp. in which they have been observed but this remains to be demonstrated. Viral endosymbionts have been reported in other protozoan species such as *Entamoeba histolytica* (Diamond and Mattern 1976).

The encysted trophozoite generally adhered closely to the cyst wall. However, many gaps were seen between the organism and the cyst wall and were more pronounced toward the posterior end of the parasite. This space has been termed a lacunar system (Feely *et al.* 1984). We do not feel that this describes the space properly, since the word lacuna suggests the existence of a network of spaces which is not evident here. In our opinion, peritrophic space describes this area more appropriately.

Ectoplasmic vacuoles appear to be ubiquitous in *Giardia* cysts (Nemanic *et al.* 1979; Sheffield 1979) and have also been reported in trophozoites (Friend 1966; Bockman and Winborn 1968). Feely *et al.* (1984) employed freeze fracture techniques to study these structures. Friend (1966) suggested that they participated in formation of the cyst wall, while Bockman and Winborn (1968) demonstrated the uptake of exogenous ferritin by these structures, suggesting that they were endocytotic in nature. Though both of these suggestions may have credence, the presence of these ectoplasmic vacuoles in the encysted *Giardia* sp. suggests additional functions for these structures. During the present study, these vacuoles were observed in untreated cysts and during the early stages of excystation only. Newly emerged trophozoites were practically devoid of any intracytoplasmic vacuoles. They were rarely seen even 30 min after incubation, a period long enough for cytokinesis to occur and daughter trophozoites to appear. These observations are consistent with our suggestion that the ectoplasmic vacuoles play a role in excystation. Lindmark (1980) and Feely (1982) demonstrated acid phosphatase activity within a portion of these vacuoles in both *Giardia lamblia* trophozoites and cysts. This finding supports the hypothesis that the cyst wall is digested by extracellular enzymatic agents synthesized internally and secreted by the enclosed organism. Coggins and Schaefer (1984) and Bingham and Meyer (1979) described a mucous material that was secreted from within the cyst as the trophozoite emerged. This substance may be composed of enzyme and decomposed cyst wall material and have a role in excystation.

One of the more interesting results of the present study was the presence of small dense vesicles on the surface of newly emerging trophozoites. The origin of these vesicles was undoubtedly cytoplasmic, as was shown, but their function remains unclear. Since they were seen only during excystation, these vesicles are clearly a part of the excystation process. The formation of the above mentioned vacuoles and cytoplasmic vesicles in the peritrophic space is perplexing, since Golgi bodies have not been described in the genus *Giardia* and an extensive network of endoplasmic reticulum was seen only after excystation had been completed. Acid phosphatase is generally considered a product of the Golgi body although *Giardia* spp. and several other protozoa lack Golgi while still exhibiting acid phosphatase activity (Martínez-Palomo 1982; Feely *et al.* 1984; McLaughlin and Aley 1985).

Results obtained in this experiment support our earlier observation (Coggins and Schaefer 1984) that cytokinesis does not

occur until after parasite emergence from the cyst. Newly emerged trophozoites were described by Coggins and Schaefer (1984) as round to oval and lacking the typical *Giardia* sp. morphology. The functional status of the sucking disk and lateral flange was unclear. Using transmission electron microscopy, the disk had a highly structured appearance as seen by the concentricity and highly ordered arrangement of microtubules and dorsal ribbons. Newly emerged trophozoites had an abundance of granular endoplasmic reticulum. These structures were not observed in the cyst stage of this study but they have been reported by previous workers (Feely *et al.* 1984). We found no Golgi or mitochondria within these organisms at any stage during excystation. This is consistent with numerous reports cited earlier on the ultrastructure of *Giardia* species.

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